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13. Abstract ***(Maximum 200 Words)*** ***(abstract should contain no proprietary or confidential information)***

The goal of this project was to elucidate how breast cancer metastases manage to survive and grow in draining lymph nodes surrounded by highly immuno-reactive cells. In animal models, we have observed that while most lung metastases are susceptible to infiltration by lymphocytes, some metastases form very compact nodules that are hypovascular and contain very little extracellular matrix. These compact metastases are very resistant to lymphocytic infiltration. We therefore hypothesized that lymph node (LN) metastases, in order to resist attack by nearby lymphocytes, grow with this compact morphology. While our animal model of B16 melanoma LN metastases demonstrated that both type of metastases (i.e., compact and non-compact (called "loose") develops, metastases in four out of six positive nodes from breast cancer patients clearly exhibited the compact phenotype. Thus, we have found clear evidence that LN metastases in humans often grow with a compact morphology which, for yet unknown reasons, protects the metastases from lymphocytic infiltration. This may explain why the metastases are able to grow in the LN and we speculate that analysis of LN metastases with respect to morphology (compact versus loose) may serve as a prognostic factor. Finally, we are investigating whether it will be possible to break the "stealth" of the compact metastases, allowing lymphocytes and dendritic cells to gain access to the malignant cells. We anticipate that better contact between tumor cells and dendritic cells/lymphocytes will lead to the induction of strong, anti-tumor responses.

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INTRODUCTION:

Draining lymph nodes (DLNs) are the key sites for priming of anti-tumor cytotoxic T lymphocytes (CTL). Despite this important immunological function, DLNs are often the first site of metastasis in breast cancer. If the DLNs contain anti-breast cancer CTL, why are breast cancer metastases able to grow at these sites, and why is it that CTL, being so close, are hardly ever seen in contact with the malignant cells? We hypothesized that anti-tumor CTL develop in DLNs, but that breast cancer metastases are allowed to grow in DLNs because the CTL are not able to reach metastases in this compartment. This might be due one (or both) of the following:

1 While unstimulated T-cells express high levels of L-selectin, primed T-cells (CTL) down-regulate their expression of this adhesion molecule. This enables them to traffic to and seek their antigen in non-lymphoid tissues, but impairs their ability to home back to lymph nodes. However, homing of CTL to metastases in lymph nodes might require the expression of L-selectin. Thus, low expression of L-selectin by CTL may inhibit their traffic "back" to metastases in the DLNs.

2 Breast cancer metastases in regional lymph nodes may have an "infiltration-resistant" phenotype (named "compact" metastases as opposed to infiltration –permissive metastases, termed "loose" metastases). If so, CTL might be able to get close to the DLN metastases (e.g., to the stroma surrounding the nodules of malignant cells), but they will not be able to infiltrate them. The scope of this study was to test one or both of these hypothesis.

BODY:

We have previously shown that lung metastases exist in two different phenotypes, namely a compact type which is infiltration-resistant and contains very little extracellular-matrix (ECM) and vasculature, and a loose type, which is permissive to

infiltration and contains abundant ECM and vasculature (1-8). Based on this experience, we have focused mainly on hypothesis #2. Our first goal was to establish an animal model of LN metastases. We therefore injected B16-F10 cells sc into animals and analyzed draining LN for micro metastases 10 days later. To our surprise, no metastases were found. Realizing that B16-F10 is selected to generate pulmonary metastases, we repeated the experiments with the parental B16-F1 cell line. This cell line produced LN metastases in all animals. Next, we analyzed the phenotype of the B16 metastases. Somewhat surprisingly, many of the B16 metastases displayed the loose morphology and contained as much extracellular matrix (judged by immuno-staining of laminin) as the surrounding normal LN tissue. Apparently, these tumors grow by infiltration of the normal LN tissue with lots of contact between malignant cells and cells of the LN (Fig 1). Thus, these metastases offered plenty of chances for the immune cells to recognize and react to the B16 cells. It, remains to be tested whether these animals develop specific immunity against the B16 cells. Interestingly, some of the B16 metastases did display the compact morphology, i.e. they contained very little laminin and the were almost totally devoid of infiltrating host lymphocytes (Fig 2).

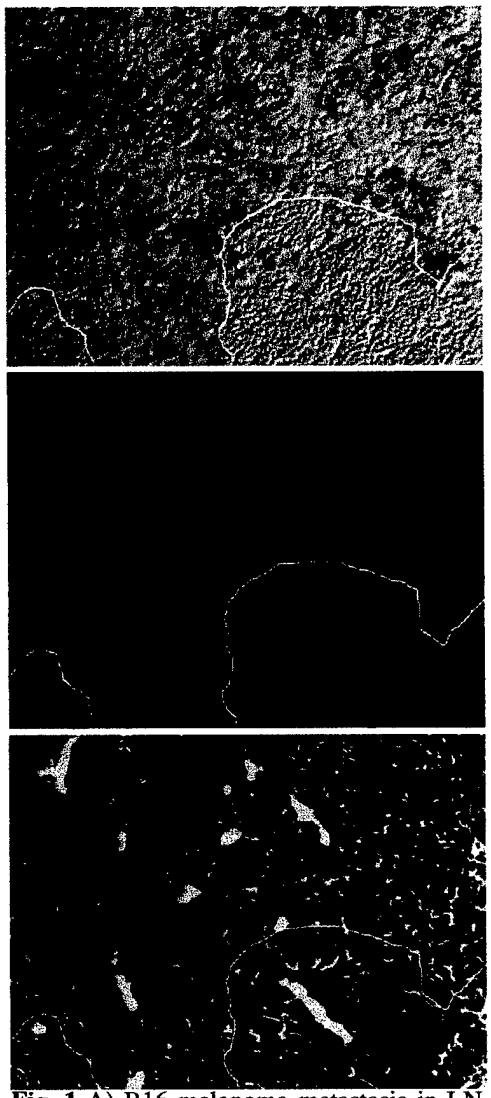

Fig. 1 A) B16 melanoma metastasis in LN. The yellow out-line marks the border between tumor and lymphoid tissue. B) Staining for CD3+ cells (red dots) reveals that these cells are present in this tumor. C) Laminin-staining (green) reveals that this tumor is of the loose type (due to high laminin content).

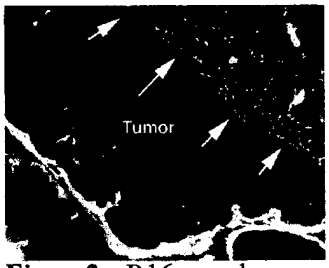

Fig. 2 B16 melanoma metastasis in LN. Staining for CD3+ cells (red dots) and Laminin (green) reveals that this tumor is of the compact type (due to high laminin content) with low infiltration by CD3 + cells. Note the sharp edge of this compact tumor (arrows).

It remains to be seen whether these metastases, being sequestered from the LN cells, eventually would survive longer that the infiltrative, loose metastases.

The analysis of metastases in lymph nodes from six breast cancer patients turned out to be much more interesting, in that the metastases in 4 out of these displayed a clear compact morpholgy, i.e. they contained relatively little laminin, they grew with a sharp line of demarcation to the surroundings and they did not seem to permit infiltration by the nearby leukocytes (Fig. 3-4). The last two samples could not be evaluated since the metastases had totally replaced the normal lymphoid tissue, i.e. the contact-area between tumor tissue and LN tissue could not be identified. Thus, the lack of contact between the malignant cells and the immuno-reactive leukocytes (especially DCs) of the LN may preclude the development immune reactions against they tumor and may explain why breast cancer metastases are able to survive and grow in draining lymph nodes. We also hypothesize that if metastases displaying the compact phenotype are less likely to induce an immune response than metastases displaying the loose morphology, analysis of the phenotype (i.e. compact versus loose) of a patients LN metastases may serve as a

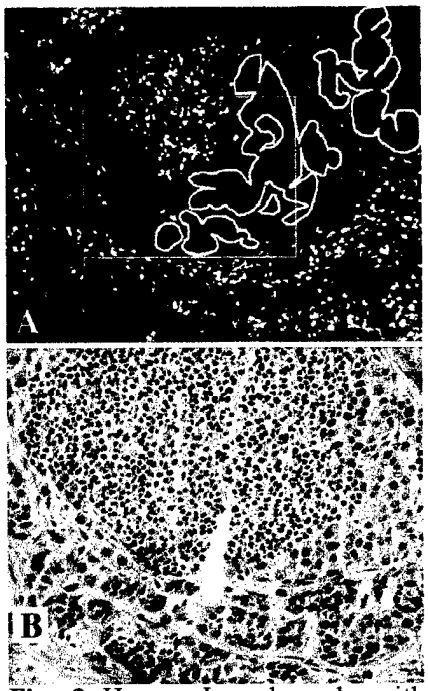

Fig. 3 Human Lymph node with metastases. A) Numerous CD3+ lymphocytes (white dots) are seen. These are found in laminin-rich areas (gray). Tumor nodules, are outlined in white. Note lack of laminin in tumor areas. **B)** Enlargement of area from serial section corresponding to rectangular area in A. Note the sharp demarcation between lymphocytes and tumor areas (H&E).

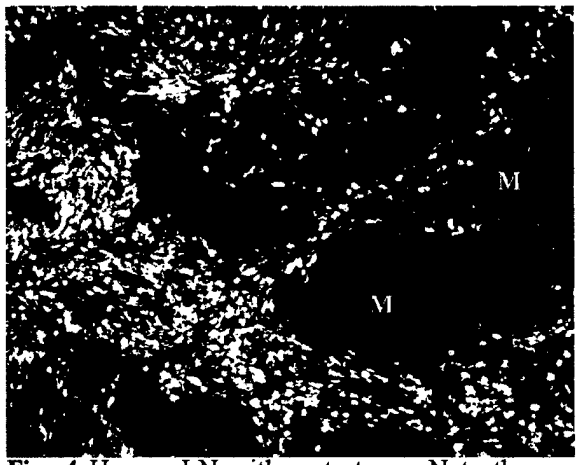

Fig. 4 Human LN with metastases. Note the co-localization between CD3 + lymphocytes (white dots) and laminin (gray). Mote lack of lymphocytes and laminin in metastases (M).

prognostic factor.

Lastly, we speculated that if one could break the stealth of the compact metastases and make them susceptible to infiltration by DCs and lymphocytes from the surroundings, this may allow for the recognition of tumor antigens and initiation of anti-tumor responses. One way to accomplish this could be to use adoptively transferred, IL-2-activated natural killer (A-NK) cells which, as demonstrated by our recent findings, are able to infiltrate even the compact type of metastases (Fig. 5). If these A-NK cells can reach lymphoid organs and metastases in these, they may be able to kill at least some tumor cells and thereby providing antigens for the immune system. In addition, other leukocytes, e.g., dendritic cells, may be able to follow the A-NK cells into the compact metastases and pick up release antigenic material. In support of this hypothesis, we able to show that adoptively transferred A-NK cells can reach lymphoid tissues and that

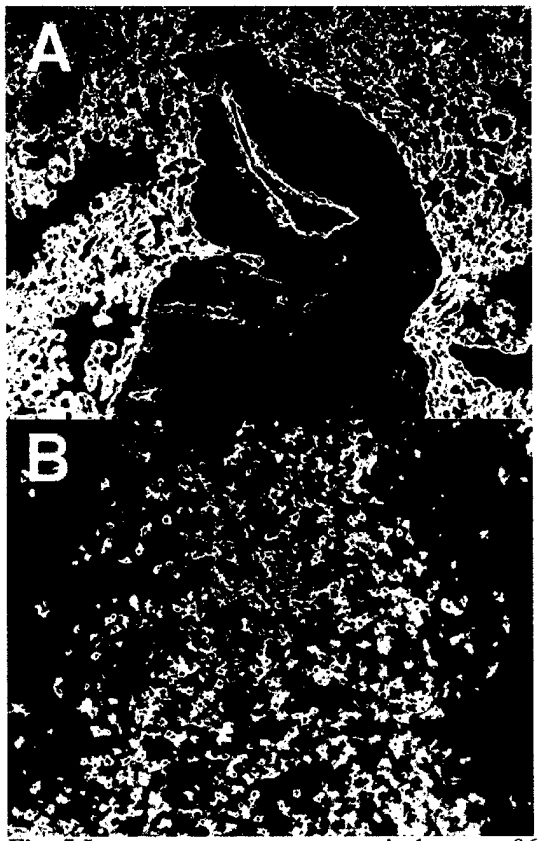

Fig. 5 Large, compact metastases in lungs at 96 hours after injection of A-NK cells. A) Laminin-staining (gray) shows very little staining in tumor, but substantial staining of normal lung tissue. B) Staining for A-NK cells (white) shows impressive infiltration of this compact tumor.

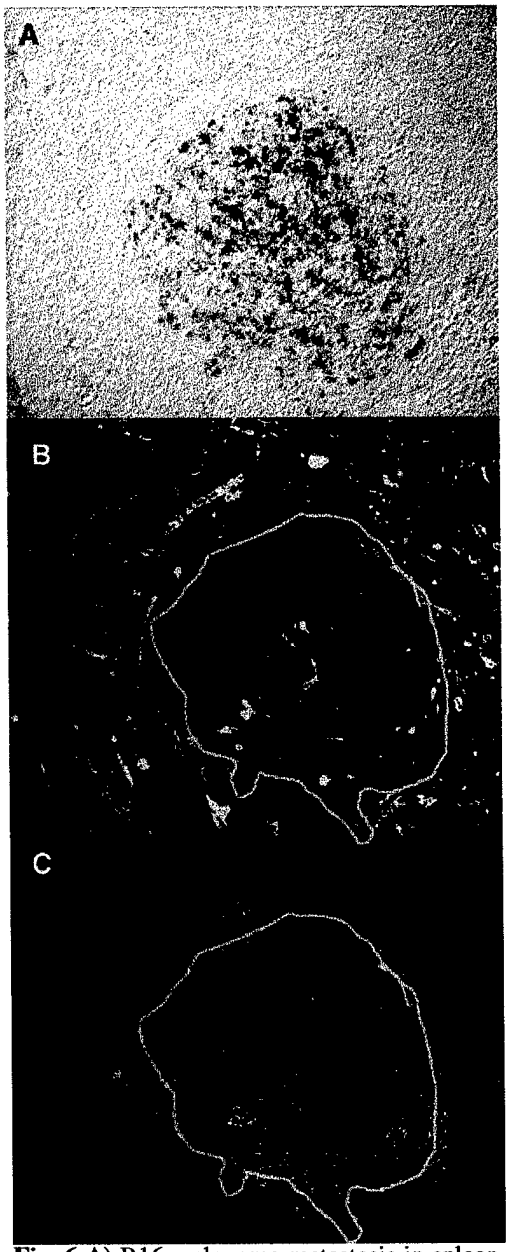

Fig. 6 A) B16 melanoma metastasis in spleen. B) Laminin-staining (green) reveals that this tumor is largely compact (due to low laminin content). C) Staining for A-NK cells (red dots) reveals that A-NK cells are able to reach and infiltrate compact metastases in lymphoid tissues.

they can infiltrate compact metastases in these tissues (Fig. 6). A proposal to further test this hypothesis, i.e., to see if the A-NK cells can really break the stealth of the compact tumors, has recently been submitted as an IDEA proposal to the DoD.

KEY RESEARCH ACCOMPLISHMENTS:

- B16-F10 tumor cells do not form LN metastases following s.c injection.
- B16 F-1 tumor cells form LN metastases. Most of these display, in contrast to B16-F1 metastases in other, non-pulmonary organs, the loose phenotype.
- Some B16-F1 LN metastases display the compact phenotype, i.e. they contain very little ECM and few infiltrating host leukocytes.
- Metastases in LN from four of six breast cancer patients all displayed the compact morphology, i.e. they contained very few vessels and very little laminin. Also, these metastases did not allow for the infiltration by leukocytes from the surroundings.
- Adoptively transferred A-NK cells are able to infiltrate compact metastases.

- Adoptively transferred A-NK cells are able to reach lymphoid tissues and to infiltrate compact metastases in these.

REPORTABLE OUTCOMES:

Funding applied for based on work supported by this award:

A proposal to test the hypotheses that 1) analysis of LN metastasis morphology (with respect to compact/loose phenotype) may serve as a prognostic factor and 2) that adoptively transferred A-NK cells may be used to break the stealth of the compact tumors, has recently been submitted as an IDEA proposal to the DoD (Proposal log number BC021913: How Do Breast Cancer Metastases Manage To Grow And Survive In Draining Lymph Nodes?).

CONCLUSIONS:

We have found that many human breast cancer metastases to lymph nodes display a compact morphology, i.e., they contain very little extracellular matrix and few vessels. They form a sharp line of demarcation to the surrounding tumor stroma or normal lymphoid tissue. They contain very few if any host leukocytes. We postulate that this stealth of the LN metastases protects them from recognition by the immune system, allowing their survival and growth in the draining lymph nodes. If this is true, patients who's LN metastases are of the compact type may react less efficiently against their tumor and therefore have a worse prognosis than patients who's LN metastases are of the loose, infiltration-permissive phenotype. Thus, analysis of LN metastases phenotype with respect to loose/compact morphology, may serve as a prognostic factor. Preliminary studies to evaluate the ability of adoptively transferred A-NK cells to break the stealth of compact metastases developing in lymphoid tissues indicates that a) the A-NK cells reach

the lymphoid tissues and b) that they have the ability to infiltrate compact metastases located in these tissues.

"so what":

We find these novel observations very exiting and of high significans for patients with node positive breast cancer. We strongly hope to be able to test the above stated hypotheses in a future projects (a proposal to do so has recently been submitted to the DoD).

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APPENDICES:

None